

# Problems and Challenges in the Cellulose to Cellulase Fermentation

M. Mandels and R. E. Andreotti\*

In recent years there has been a marked interest in enzymatic saccharification of cellulose, particularly due to the availability of active cellulase from *Trichoderma* capable of extensive hydrolysis of insoluble cellulose. Several recent symposia have evaluated the process and examined the severe technical and economic constraints on it, including limited availability of suitable substrates, necessity of costly pretreatments, and high costs of the enzyme. Interest and research have, however, continued because cellulose is the only renewable resource available in large quantities. In this paper the authors discuss some of the constraints and problem areas of the fermentation to produce cellulase in quantity.

## Materials and methods

**Cultural.** *Trichoderma* QM9414 was maintained on potato dextrose agar slants. The basal medium for growth and enzyme production contained, per litre,  $\text{KH}_2\text{PO}_4$ , 2.0g,  $(\text{NH}_4)_2\text{SO}_4$ , 1.4g, Urea 0.3g,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.3g,  $\text{CaCl}_2$  0.3g,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  5.0mg,  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  1.6mg,  $\text{ZnSO}_4$  1.4mg, and  $\text{COCl}_2$  2.0mg. The cellulose source was Solka Floc (SW40) a 40 mesh hammer-milled, fibrous, pure cellulose pulp (Brown Co, Berlin, New Hampshire). Proteose peptone (Difco) and Tween 80 (polyoxyethylene sorbitan monooleate, Fisher) were added to the medium as noted. Shake flasks for inoculum were made up by using the fermentation medium (0.75% cellulose), inoculated with conidia and grown for three days at 27°C on a reciprocating shaker. Inoculum volume was 2-5%.

Submerged culture experiments were carried out in New Brunswick Scientific Co, Magnaferm Fermenters Model MA114. This fermenter utilises a 15 litre glass vessel equipped with a magnetically-driven, triple-propeller stirrer and a mechanical foam breaker. Temperature, dissolved oxygen, and pH were controllable, and were continuously recorded during the experiments. The pH was controlled by using 2N NaOH and 2N HCl. Temperature was controlled at 27°C, aeration was at 15 pounds pressure, 1-2 litre/min flow (0.1-0.2vvm), impeller was at 200-250rpm. During active growth the air flow and agitation were increased as required to maintain positive dissolved oxygen and good mixing.

**Extracellular Components.** Cellulase assays were carried out on culture filtrates at pH 4.8 and 50°C and results are expressed as international units (U) equal to micromoles of reducing sugar as glucose released per minute. Substrates and time of incubation were: filter paper (saccharifying) cellulase, Whatman No. 1 filter paper, 33mg/ml, 60 minutes and endo-B-1, 4-glucanase (CMC'ase) carboxymethyl cellulose, degree of substitution 0.5 (Hercules), 5mg/ml, 30 minutes and endo-B-1, 4-glucanase mg of reducing sugar as glucose produced when 50mg of absorbent cotton was incubated with 1ml of culture filtrate for 24 hours. The reducing sugar was measured by a dinitrosalicylic acid procedure<sup>21</sup>. Soluble

protein in the culture filtrate was precipitated with 5% trichloroacetic acid and measured by the folin procedure<sup>19</sup>. Bovine serum albumin was used as a protein standard. Protease was measured as the optical density at 595nm of the colour released from hide

Table 1. Cellulolytic Organisms

Bacteria	Reference
<i>Cellvibrio fulvus</i> —aerobe, mesophile	
<i>Cellvibrio gilvus</i> —aerobe, mesophile	32
<i>Cellvibrio vulgaris</i> —aerobe, mesophile	
<i>Cellulomonas</i> —aerobe, mesophile	29
<i>Pseudomonas fluorescens</i> —aerobe, mesophile	34
<i>Ruminococcus</i> —anaerobe, Rumen	17
<i>Clostridium thermocellulaseum</i> —anaerobe, thermophile	11, 18
<b>Actinomycetes (aerobic)</b>	
<i>Streptomyces</i> QMB814—mesophile	25
<i>Thermoactinomyces</i> —thermophile	4, 5
<i>Thermomonospora curvata</i> —thermophile	33
<i>Thermomonospora fusca</i> —thermophile	10
<b>Fungi (aerobic)</b>	
<i>Aspergillus niger</i> —mesophile	Commercial
<i>Trametes sanguinea</i> —mesophile	
<i>Poria</i> —mesophile	
<i>Myrothecium verrucaria</i> QM460—mesophile	
<i>Pestalotiopsis westerdijkii</i> QM381—mesophile	
<i>Penicillium iirizensis</i> QM9624—mesophile	6
<i>Penicillium funiculosum</i> —mesophile	27, 28
<i>Penicillium variabile</i> —mesophile	1
<i>Polyporus versicolor</i> —mesophile	
<i>Polyporus tulipiferae</i> —mesophile	16
( <i>Irpea lacteus</i> )	
<i>Fusarium solani</i> —mesophile	38
<i>Trichoderma viride</i> —mesophile	
<i>Trichoderma lignorum</i> —mesophile	
<i>Trichoderma koningii</i> —mesophile	
<i>Sporotrichum pulverulentum</i> QM9145—thermophile	12
( <i>Chrysosporium lignorum</i> , <i>Phanerochaete chrysosporium</i> )	
<i>Sporotrichum pruinosum</i> QM826—thermophile	
( <i>Chrysosporium pruinosum</i> )	
<i>Sporotrichum dimorphosporum</i> QM806—thermophile	
<i>Sporotrichum thermophilum</i> QM9382—thermophile	9, 35
<i>Chaetomium thermophilum</i> QM9381—thermophile	26, 35
<i>Thermoascus aurantiacus</i> QM9383—thermophile	26, 35

powder azure in 1 hour at 50°C, pH 4.8.

**Cellular Components.** A 50ml aliquot of the culture was filtered by suction on a tared 5.5cm glass filter paper (Reeve Angell) washed with water and dried overnight at 80°C, then weighed to measure the dry weight which includes mycelium and residual cellulose. The mat was peeled off the glass paper, soaked overnight in 5ml of distilled water, then extracted 3 times at 100°C for 10 minutes in 1N NaOH. Mycelial protein was measured on the combined extracts by a biuret procedure<sup>14</sup>. Bovine serum albumin was used as a protein standard. The residue was washed with water and extracted twice at room temperature for 30 minutes with 10ml of 67%  $\text{H}_2\text{SO}_4$ . A phenol sulphuric analysis of the combined acid extracts was used to measure total carbohydrate = residual cellulose<sup>14</sup>. Alternatively cellulose was estimated by assuming that the mycelial protein represented 40% of the cell weight and subtracting this value from the dry weight<sup>1</sup>.

## Strain selection

In the authors' experience all active development of practical processes for saccharification is based on the *Trichoderma* enzyme system. Frequently suggestions are made that too much attention has focused on *Trichoderma* and that other sources of cellulase should be developed. This seems quite reasonable. Many microorganisms are actively cellulolytic. A few that have received recent attention are listed in Table 1.

However, rapid growth on, and decomposition of cellulose, and production of high levels of enzyme hydrolysing soluble cellulose derivatives are not adequate criteria for selecting organisms to be used as a source of cellulase. Likewise the production of small amounts of sugar from insoluble cellulose by extracellular enzyme preparations in short time assays cannot be extrapolated to predict results of more extended saccharification, because such results may be based on the hydrolysis of the limited fraction of amorphous cellulose present in the substrate.

The hydrolysis of a multiple insoluble substrate (cellulose) by a multiple enzyme (cellulase) proceeds in stages. The first is a fairly rapid hydrolysis of the most susceptible portion of the cellulose which can be carried out by either exo- or endo-B-glucanases. A good many organisms produce extracellular endo-B-glucanases that can hydrolyse cotton (the most crystalline and resistant form of cellulose) up to about 1%.

\*Mary Mandels and Raymond E. Andreotti, Enzyme Technology Group, Pollution Abatement Division, U.S. Army Natick Research and Development Command, Natick, Mass 01760 USA.

As hydrolysis proceeds, the rate slows down as the residue becomes increasingly crystalline and resistant. If the cellulase is incomplete the rate may become practically zero at this point. Hydrolysis of crystalline cellulose requires synergistic action between endo- and exo- $\beta$ -glucanases and is accelerated by  $\beta$ -glucosidase and possibly by other cellulase components. Contrary to the situation when a single enzyme acts on a soluble well defined substrate, initial rates give little information about the properties of the enzyme.

To detect a complete cellulase, hydrolysis must continue until some of the crystalline cellulose is hydrolysed. Furthermore, a

meaningful comparison of cellulase preparations can only be made on the basis of the same percent conversion of the substrate. Twice as much enzyme will give the same conversion in half the time, but it will not produce twice as much sugar in equal time.

Strain selection must therefore be based on a reasonably extensive hydrolysis of insoluble cellulose, and for quantitative results the unit values must be based on equal percent conversions. When this is done, using filter paper as a substrate, and 4% conversion as the basis for calculation of units, very few organisms yield broths having more than 0.3 filter paper units/ml (filter paper activity 1.6). Wild strains of

*Trichoderma* such as QM6a produce 0.5 U/ml or more in shake flask culture and the mutant strains QM9123 and QM9414 produce up to 2.0 U/ml (Table 2). When these filtrates are used to saccharify milled cellulose materials in 5-20% slurries, conversions of 50% or more can be attained in 24 hours.

The reason that so much effort has focused on *Trichoderma* is that no one has ever published real saccharification data (ie reasonably extensive conversion of a reasonably concentrated realistic substrate) based on a cellulase preparation derived from any source other than *Trichoderma*. Therefore the authors are concentrating on optimising the fermentation production of

**Table 3. Problem areas in the *Trichoderma* Cellulase fermentation**

Cellulase—growth vs enzyme production

Insoluble

Low bulk density

Absorbs cellulase

Cellulase—growth vs enzyme production

Induced enzyme

Repressed by soluble sugars

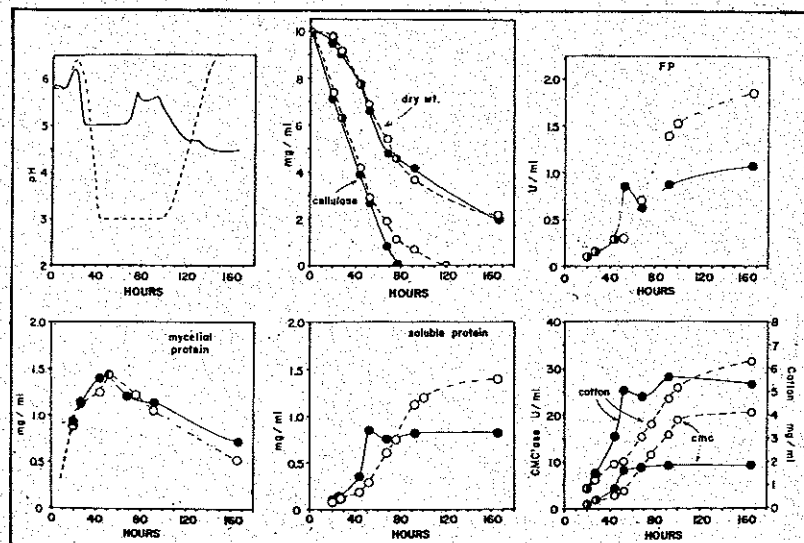
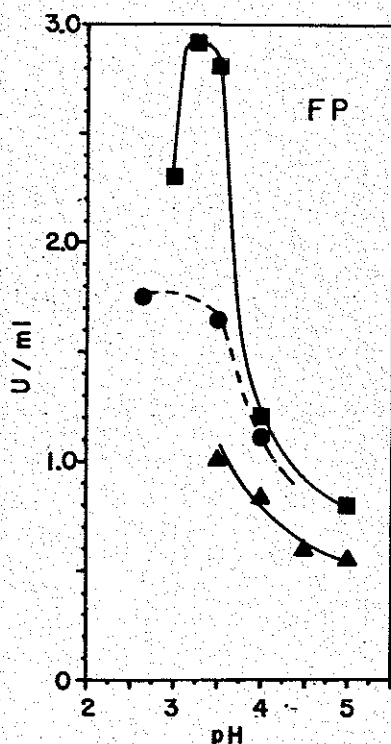
pH control is critical

Low specific activity

Produced late in fermentation

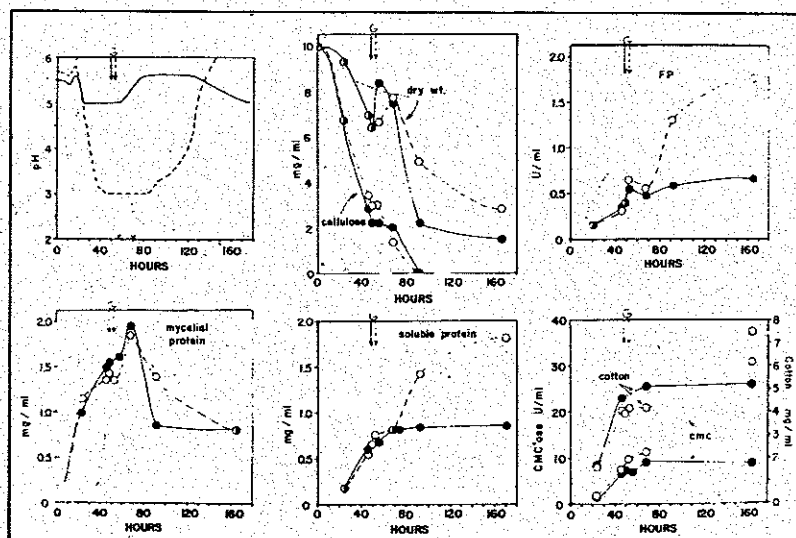
Low in  $\beta$  glucosidase

**Figure 1. Effect of pH Control on Cellulase Production.** Maximum filter paper cellulase is shown for a 7-day fermentation. pH control with 2N NaOH and 2N HCl.  $\bullet$ — $\bullet$  0.75% cellulose, 0.075% peptone, 0.2% Tween 80, 10% mycelial inoculum. pH 2.6 uncontrolled. Others natural fall to indicated pH.  $\blacktriangle$ — $\blacktriangle$  1.0% cellulose, 0.05% proteose peptone, 0.1% Tween 80, 2% mycelial inoculum. pH 3.5 natural fall to control point. Others controlled at indicated pH from inoculation.  $\blacksquare$ — $\blacksquare$  2.0% cellulose, 0.2% peptone, 0.1% Tween 80, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> raised to 0.21%. 2% mycelial inoculum. pH controlled not to fall below indicated level (no acid used).



**Figure 2. Growth and Enzyme Production on 1% Cellulose at pH 3.0 and 5.0.** 1% Cellulose 0.1% proteose peptone, 0.1% Tween 80, 2% Mycelial Inoculum, 3 days old.

	N NaOH ml/litre	Productivity U/litre.hr <sup>-1</sup>
$\circ$ — $\circ$ pH>3.0	5.4	11.1
$\bullet$ — $\bullet$ pH>5.0	10.4	6.5



**Figure 3. Effect of Adding Glucose during Fermentation of 1% Cellulose at pH 3.0 and 5.0.** 1% Cellulose, 0.1% proteose peptone, 0.1% Tween 80, 2% Mycelial Inoculum, 3 days old.  $\circ$ — $\circ$  pH>3.0, 0.5% glucose added at 53 hours.  $\bullet$ — $\bullet$  pH>5.0, 0.5% glucose added at 49 hours.

	<i>N</i> NaOH ml/litre	Productivity U/litre hr <sup>-1</sup>
pH	before	after glucose addition
3.0	3	12
5.0	7	13

this enzyme. Because of the low bulk density and recalcitrance of cellulose large quantities of enzyme are required for saccharification and enzyme cost is a major factor in the economics of the process.

#### Optimisation of media and fermentation conditions

Optimisation of any fermentation for production of a desired biological product will involve many problems related to strain selection, media composition, inoculum, and conditions of growth. The *Trichoderma* cellulase fermentation must of course be optimised along these lines, but it also has some unusual problem areas related to the insolubility and multiplicity of the substrate

and the multiplicity of the enzyme system (Table 3).

1. Cellulase is an induced enzyme in *Trichoderma* produced only when the fungus is grown on cellulose, other glucans containing  $\beta$ -1,4 linkages, cellobiose, lactose, or sophorose. This means that for practical purposes the fungus must be grown on cellulose to produce cellulase. The use of a slowly metabolised insoluble substrate creates challenging problems for the fermentation.

2. Cellulose strongly adsorbs cellulase, the fermentation product. Insoluble impurities in crude cellulose such as lignin also adsorb cellulase. The authors have not found

satisfactory procedures for desorbing and recovering enzyme from undigested solids.

3. The production of cellulase is repressed by soluble sugars or other readily metabolised substrates.

4. Control of pH is critical. The pH of the *Trichoderma* medium is about 5.7 after autoclaving. As the cellulose is consumed,  $\text{NH}_4^+$  is also consumed and pH falls steeply. On cellulose concentrations of 0.75% pH falls to about 2.7 and then slowly rises. Yields of cellulase are high. At cellulose concentrations of 1% or greater, if pH is uncontrolled, enzymes are inactivated by the severe acid conditions which develop. Therefore it is necessary to control pH not to fall below 3.0-3.5 to prevent enzyme inactivation, and then higher yields are attained than on 0.75% cellulose<sup>6</sup>. However, at all concentrations of cellulose, if pH is controlled not to fall below 4.0, 4.5, or 5.0 growth is excellent, but cellulase yields are reduced, and the higher the level at which pH is controlled the greater the reduction in cellulase (Figure 1).

The reason for the yield reduction at high pH is not clear. One possibility is enzyme repression due to more rapid growth at higher pH levels. Brown and Halsted<sup>7</sup> have shown that the growth rate on glucose is markedly reduced as pH falls from 4.0 to 2.7 and is essentially zero at pH 2.5. Likewise we find that on cellulose if pH is controlled at 3.0-3.5 from time of inoculation, growth is poor, but then enzyme yields are also poor.

When 1% cellulose cultures were compared with an initial pH of 5.7 and a natural fall to control at 5.0 or 3.0 (Figure 2) good growth occurred in both cultures, but cellulase production was markedly superior at 3.0. It took 40 hours to reach the control point of 3.0 and by that time considerable growth of mycelium and consumption of cellulose had already occurred.

Still up to 52 hours, the growth rate as indicated by consumption of cellulose and production of mycelial protein was somewhat greater at 5.0 and at 52 hours more than twice as much cellulase had been produced at 5.0 as at 3.0. At this time when biomass (mycelial protein) was at its peak and 70% of the cellulose had been consumed in both cultures, production of cellulase nearly ceased at 5.0 just when one would expect the fungus to be derepressed, and when in fact the period of most active enzyme secretion began in the 3.0 culture.

From 52-166 hours cellulase measured on filter paper, CMC, or cotton increased not more than 20% in the 5.0 culture, but increased 3-6 fold in the 3.0 culture. Thus repression at pH 5.0 seems an unlikely explanation for the reduced yields.

The release of enzyme at 3.0 is not due to the death and dissolution of the culture. *Trichoderma* can metabolise glucose quite rapidly at pH 3.0. Glucose did not accumulate in either culture during fermentation despite the rapid disappearance of cellulose. When 0.5% glucose was added to a 1% cellulose culture at 3.0 (Figure 3) it was consumed in 17 hours. At 27° the hydrolysis of either crystalline or amorphous cellulose is most rapid at pH 3.5-4.8 without a sharp optimum. It is also rapid at 3.0 but is slower at 6.0 (Tables 4 and 5).

Another possibility would be proteolytic degradation of secreted enzymes at pH 5.0, but this also is unlikely. The culture filtrates of Figure 2 and 3 were assayed for proteolytic

Table 2. *Trichoderma* strains on Natick collection

QM No	ATCC No	Type	Cellulase FP Units per ml
6a	13631	Wild Strain	0.5-0.7
9123	24449	Enhanced Cellulase Mutant Derived from QM6a	1.0-1.2
9414	26921	Enhanced Cellulase Mutant Derived from QM9123	1.5-2.0
9136	26920	Cellulase Negative Mutant Derived from QM6a	0

Cellulase activity in shake flasks grown on cellulose media.

These strains are available from Dr Emory G. Simmons, US Army Development Centre Culture Collection of Fungi (QM), Dept Botany, University of Massachusetts, Amherst, MA 01002 USA, or from The American-Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, MD 20852 USA.

Table 4. Effect of pH on Hydrolysis of Microcrystalline Cellulose (Avicel) at 27°

Time Hr	Enzyme U/ml	% Hydrolysis			
		pH 3.0	pH 3.5	pH 4.8	pH 6.0
2	2.0	4.0	4.5	4.4	1.4
2	1.0	2.4	2.5	2.4	1.2
2	0.5	1.9	1.9	1.9	1.0
20	2.0	8.3	8.8	8.1	3.5
20	1.0	5.9	6.4	6.3	2.9
20	0.5	3.9	4.5	4.7	1.9
100	2.0	15.0	16.8	15.8	7.6
100	1.0	10.7	11.7	12.0	4.8
100	0.5	7.8	8.2	8.5	3.5

Conditions 10% Avicel, 0.05M Citrate Buffer

Enzyme = *Trichoderma* QM9414 culture filtrate from a 2% Cellulose fermentation at 7 days to give 2.0, 1.0, or 0.5 filter paper cellulase units per ml.

% Hydrolysis based on Average of Reducing Sugar and Weight Loss

Table 5. Effect of pH on Hydrolysis of Ball Milled Cellulose (BW 200) at 27°

Time Hr	Enzyme U/ml	% Hydrolysis			
		pH 3.0	pH 3.5	pH 4.8	pH 6.0
2	2.0		5.2	5.1	3.7
2	1.0		4.0	3.9	3.2
2	0.5		3.2	2.9	2.0
20	2.0		14.6	13.3	6.8
20	1.0		10.0	9.3	5.0
20	0.5		7.0	7.0	4.0
100	2.0	27.6	29.3	27.2	14.8
100	1.0	19.1	19.6	19.5	10.6
100	0.5	12.7	14.4	14.2	7.4

activity against the insoluble substrate, hide powder azure (Table 6). The levels were high at 3.0, low at 5.0. In fact, the authors have always found high protease levels in the most active cellulase filtrates, and have found no evidence that these proteases are active on cellulase.

One could speculate that the higher

protease levels are part of a general release of cell contents and may even play a role in such release, but this has not been investigated. As noted above, the mycelium remains healthy and metabolically active at 3.0. Furthermore the authors have not noted the release of sugars at low pH that would be expected from autolysing cells.

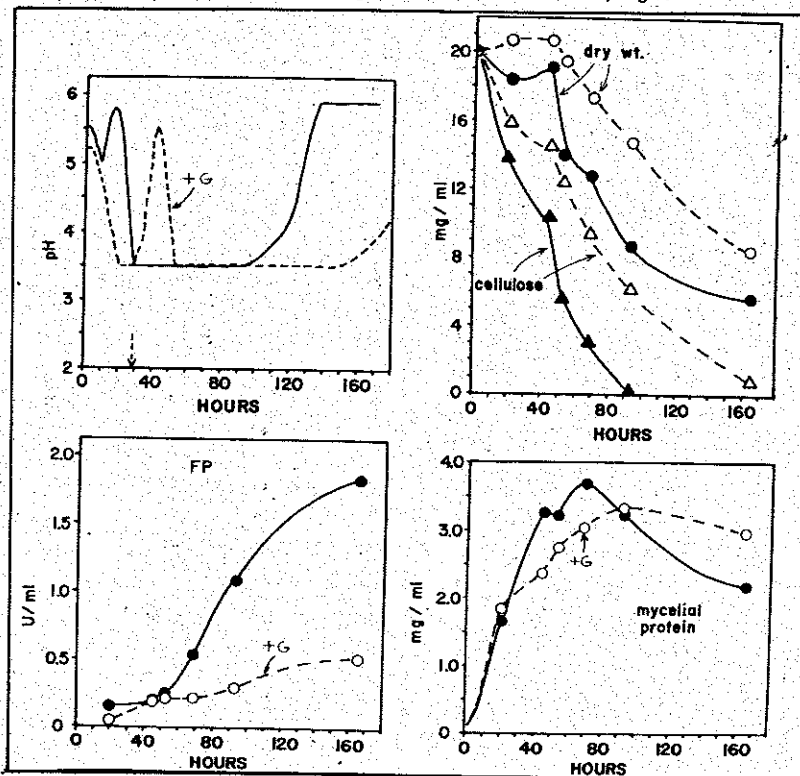


Figure 4. Effect of Addition of Glucose to 2% Cellulose Medium. 2% Cellulose, 0.2% protease, peptone, 0.1% Tween 80,  $(\text{NH}_4)_2\text{SO}_4$  0.21%, Sag 10 200 ppm, pH>3.5. 5% Mycelial Inoculum, 3 days old. •—• 2% cellulose. o---o 2% cellulose + 0.4% glucose at 0 hr.

	N NaOH ml/litre	Productivity U/litre hr <sup>-1</sup>
cellulose	21.6	11.1
cellulose + glucose		3.1
glucose phase	2.2	
cellulose phase	13.6	

Table 6. Effect of pH Control on Protease Production in Cellulose Medium

Time Hr	1% Cellulose		1% Cellulose + 0.5% Glucose	
	pH>5.0	pH>3.0	pH>5.0	pH>3.0
20	0.12	0.12	0.16	0.14
52	0.13	0.11	0.12	0.12
68	0.18	0.33	0.12	0.95
95	0.17	0.75	0.13	1.28
166	0.16	1.16	0.12	1.04

Conditions. 1% Cellulose, 0.1% Peptone, 0.1% Tween 80, 2% Mycelial Inoculum, 3 days old, 0.5% Glucose added at 50 hours (see Figures 2, 3)

Protease Activity. 20mg Hide Powder Azure (insoluble), 2.5ml Enzyme, 2.5ml 0.05M Citrate Buffer pH 4.8, Incubate 1hr at 30°, 1hr at 50°, Filter—Read OD of filtrate at 595.

Table 7. Effect of Substrate on Specific Cellulase Activities

Substrate	Concentration	Incubation Time	International U/mg Protein
Carboxymethyl Cellulose	0.5	30min	50.
Filter Paper (FP)	3.3	1hr	1.
Cotton	2.5	24hr	0.02

One international unit releases one micromole of reducing sugar measured as glucose per minute.

There thus appears to be no good explanation of the reduced yield at higher pH levels. The authors' conclusions are that in batch cultures the best regime is a natural fall to 3.0-3.5, control to prevent a lower pH, and a natural rise after cellulose is consumed. Maintaining the low pH after cellulose consumption has no effect on enzyme yield. Continuous culture is another situation. With continuous low pH, growth is retarded and enzyme yields are reduced. Good yields in continuous culture have been attained at pH 5.0<sup>22, 24, 27</sup>.

5. Cellulase has a low specific activity on insoluble cellulose, only about one international unit per mg of soluble protein when filter paper is the substrate (Table 7). This is due to the insolubility and recalcitrance of the cellulose. In the fermentation of 1% cellulose, yields of 2mg of extracellular protein (2 filter paper units) per ml have been attained. Assuming a 50% yield of biomass from the substrate, this extracellular protein represents 40% of the biomass. It is apparent that higher yields will require more cells, ie more growth and richer media. There are several possible approaches to achieve this.

a. Increasing cellulose concentration. Because of the low bulk density of cellulose this results in thick slurries and severe foaming problems, and creates difficulties in adequate mixing and aeration. The maximum working cellulose concentration is about 2% in small laboratory fermenters although submerged fermentations have been carried out on up to 8% cellulose<sup>23</sup>. At that point one is approaching a solid culture or koji fermentation.

In fact, practically all of the commercial *Trichoderma* cellulase available to date has been produced in Japan by solid culture fermentation followed by extraction of the enzyme from the culture solids<sup>26</sup>. This fermentation is of special interest to developing countries because the technology can be fairly simple, and variations of the koji process can be used to upgrade poor quality agricultural wastes to more valuable animal feed<sup>25</sup>.

The drawbacks of solid culture for enzyme production are, first the difficulties of monitoring and controlling the fermentation, and second the fact that some of the cellulase components tend to remain adsorbed on the solid residues.

Another approach has been to increase cell growth by enriching cellulose media with soluble substrates such as glucose, glycerol, or preferably soluble peptones or proteins. Low levels of such metabolites are frequently added to cellulose media and do result in shorter lags, faster growth, and increased cellulase levels. However, when the soluble substrates are increased beyond about 10% of the cellulose level, cellulase production is repressed and so growth may actually be reduced.

b. Addition of glucose to cellulose media. The authors have tried to increase biomass production by adding 0.4% glucose to 2% cellulose medium in the hope that the glucose would be rapidly consumed and build up biomass, that cellulose consumption would be repressed during this period so that cellulose would be consumed only after the consumption of glucose at which time synthesis of cellulase would be derepressed. The results were both surprising and disappointing (Figure 4).

The cellulose fermentation was typical and successful. Cellulose was rapidly consumed with a pH fall to 3.5 by 30 hours. A total of 21.5mEq of NaOH per litre was consumed to hold pH at 3.5 until 90 hours when cellulose was consumed and pH rose again. Although half the cellulose had been consumed by 45 hours, only 10% of the cellulase had been secreted into the medium at that time. A peak in biomass (mycelial protein/0.40) of 9.3mg/ml was reached at 69 hours representing a 45% yield of consumed substrate. Cellulase was secreted rapidly from 45 hours, and especially after 69 hours reaching a final yield of 1.8 filter paper units (34 CMC cellulase units) per ml at 165 hours for an overall productivity of 11U/litre · hr<sup>-1</sup>.

The effects of adding glucose were very interesting. The glucose was very rapidly consumed by about 30 hours, but 25% of the cellulose was also consumed during this period. The culture consumed 2.2mEq of NaOH per litre to control pH at 3.5 during this period. The culture consumed 2.2mEq of growth and increased biomass production was thus achieved although it had not been anticipated that so much cellulose would be consumed during the glucose consumption phase.

After the glucose was gone, the growth stopped and pH rose to 5.5 at 40 hours then fell again as cellulose consumption resumed. An additional 13.6mEq of NaOH per litre was consumed from 50-150 hours when cellulose was consumed and pH rose again. Overall growth was actually reduced and total acid production was decreased despite the increased substrate.

A peak in the biomass of 8.4mg/ml was reached at 93 hours, lower and later than the peak in the cellulose culture. The final yield of cellulase at 165 hours was only 0.5 filter paper or 8 CMC'ase units per ml, about 25% of the values for cellulose with no added glucose. Thus the final effect of adding a fairly high level of glucose to cellulose medium was to so reduce cellulase production that growth was also reduced below what it would have been on the cellulose alone.

The effect of adding glucose during the fermentation has also been investigated with 1.0% cellulose controlling pH at 3.0 and 5.0 (Figure 3). Glucose (0.5%) was added at 50 hours. The results can be compared to the similar fermentations without glucose addition (Figure 2). The added glucose was consumed in 9 hours at pH 5, 17 hours at pH 3. During the period of glucose consumption secretion of cellulase and consumption of cellulose ceased, while dry weight and biomass (mycelial protein) increased sharply. After the glucose was consumed dry weight and mycelial protein fell, cellulase consumption resumed and in the pH 3.0 culture, secretion of soluble protein and cellulase resumed.

The addition of glucose late in the fermentation thus had very little effect on the fermentation pattern of enzyme productivity, in marked contrast to the strong deleterious effect when it was added to the original medium. Even though most of the enzyme is secreted late in the fermentation, the fungus appears to be committed early to low or high enzyme production.

c. *Continuous cultures.* The problems of maintaining a high biomass are even more acute in continuous cultures. If pH is maintained low, growth and enzyme

productivity are poor. Peitersen<sup>24</sup> has investigated continuous culture of *Trichoderma* at pH 5.0, 30°C with a feed of ball milled cellulose of 4.11g/litre and with dilution rates of 0.03-0.08hr<sup>-1</sup>. Under these conditions 50-75% of the cellulose was consumed leaving 1.4g/litre at steady state. Cell protein ranged from 0.6-2.4mg/ml, enzyme from 0.1-0.3U/ml. Productivity (5-13U/litre · hr<sup>-1</sup>) was thus equal to the overall productivity of batch cultures on 1-2% cellulose of about 11U/litre · hr<sup>-1</sup> (Table 8, Figures 2, 3 and 4, Andreotti et al 1977<sup>3</sup>), but enzyme concentration in the harvest was low.

In a good 1% batch cellulose fermentation the highest productivities of about 20-25U/litre · hr<sup>-1</sup> occur between 50 and 100 hours. Nystrom<sup>23</sup> has had productivities of 40-45U/litre · hr<sup>-1</sup> on 5% cellulose. These conditions (long residence times) cannot be duplicated in a continuous culture.

d. *2 stage fermentation.* Other approaches to increasing cell mass in continuous fermentation have therefore been attempted. Mitra and Wilke<sup>25</sup> proposed a 2 stage fermentation with *Trichoderma* growing on glucose in the first (growth) stage and on cellulose in the second (induction) stage. Maximum cell productivities of 0.47mg/ml · hr<sup>-1</sup> on 0.5% glucose and 0.92mg/ml · hr<sup>-1</sup> on 1.0% glucose were attained at a dilution rate of 0.21 · hr<sup>-1</sup> giving an inlet biomass to the second stage of 2.2mg/ml from 0.5% glucose and 4.4mg/ml from 1.0% glucose.

In the cellulose fermenter, even though pH was controlled at 5.0, growth was poor and the culture washed out at dilution rates greater than 0.02 · hr<sup>-1</sup>. Doubling the inlet biomass nearly doubled enzyme productivity from 1.4 to 2.6U/litre · hr<sup>-1</sup>, but since these are much lower than attained by Peitersen<sup>24</sup> on cellulose alone it is apparent that the initial growth on glucose actually reduced overall growth due to the strong repression of enzyme production.

Glucose repression is also probably the explanation of the results of Brown et al<sup>6</sup> for continuous culture of *Trichoderma* on crude glucose containing the inducer sophorose. Despite rapid growth on glucose, cellulase was not produced at dilution rates greater than 0.03 · hr<sup>-1</sup> and maximum enzyme productivity (at D = 0.015 · hr<sup>-1</sup>) was only 1.2U/litre · hr<sup>-1</sup>.

e. *Cell recycle.* A means of avoiding glucose repression while enhancing biomass in a continuous cellulose fermentation by recycling the mycelium back into the fermenter as the broth is harvested has been proposed by Wilke et al<sup>27</sup>. The use of cellulose grown cells resulted in a dramatic improvement of enzyme productivity and of enzyme concentration in the harvest. In a semi-continuous run on 1.0% cellulose with a dilution rate of 0.02 · hr<sup>-1</sup> the harvest averaged 0.8U/ml for a productivity of 16U/litre · hr<sup>-1</sup>, 6 fold the yield in the 2 stage process described above. Although this value is only about 25% greater than Peitersen's<sup>24</sup> best yield in a simple continuous cellulose culture, the enzyme concentration in the harvest broth is increased more than 2 fold. Cell recycle looks like a promising area for further investigation.

6. Cellulase is produced late in the fermentation. On 1% cellulose with optimum pH control at 3.5 (Andreotti et al<sup>3</sup>) the fermentation can be divided into 3 phases of rapid growth, high enzyme productivity, and old age. In the first 45 hours, dry weight, the resultant of increasing mycelial weight and decreasing cellulose, has fallen about 50%. At this time 80% of the cellulase has been consumed, cell weight has reached maximum but only 20% of the cellulase has appeared.

Forty-five hours is a turning point. Enzyme production suddenly picks up. From 45-70 hours the last 20% of the cellulase is consumed, cell weight falls very slightly, and soluble protein and enzyme are secreted rapidly. 40% of the filter paper cellulase and 60% of the CMC'ase appear during this period of high enzyme productivity. From 70 to 160 hours is the old age—no cellulose remains, cell mass, the only remaining substrate declines, and the last 20% of the CMC'ase, and 40% of the filter paper cellulase appear.

Thus although the cellulase is an induced enzyme, *Trichoderma*, during growth on cellulose, appears to produce much more cellulase than is required to digest the substrate and to continue secreting enzyme after most of the cellulose is consumed. The authors believe that most of the cellulase is synthesised late in the fermentation, although the fungus is committed earlier. It is not possible that it was adsorbed on the

Table 8. Cellulase Productivities by *Trichoderma*

Batch Culture 1% Cellulose	Time Hour	Cellulase U/ml	Productivity U/litre hr <sup>-1</sup>
Whole Run (Andreotti et al <sup>3</sup> )	162	1.85	11.
Maximum Period (Andreotti et al <sup>3</sup> ) Continuous 1% Cellulose	51-99 D hr <sup>-1</sup>	0.42-1.52	23.
Simple (Peitersen <sup>24</sup> )	0.038	0.33	12.5
2 Stage 1% Glucose → Cellulose (Mitra et al <sup>25</sup> )	0.015	0.12	2.6
Cell Recycle (Wilke et al <sup>27</sup> ) Continuous 1% Glucose	0.02	0.80	16.
Sophorose inducer (Brown et al <sup>6</sup> )	0.015	0.08	1.2

Cellulase Filter Paper Units per ml (Mandels et al<sup>20</sup>) Units were estimated from the Filter Paper Activities for 1ml reported by Mitra, Wilke, and Brown.

Productivity Filter Paper Units per Litre of Culture per hour.

residual cellulose; in fact much of it appears after the cellulose is gone. Perhaps this is one reason why *Trichoderma* is a superior source of cellulase—other fungi may “turn off” the enzyme production sooner. But the long slow fermentation, and the late appearance of much of the cellulase create problems for the cost accountant or the engineer who wishes to run a continuous fermentation.

7. *Trichoderma* cellulase is deficient in  $\beta$ -glucosidase. Maximum concentrations in broths from cellulose cultures are only about 0.8 units per ml. The result is that saccharification syrups are high in cellobiose<sup>2</sup>. Because cellobiose is strongly inhibitory to saccharification, with an inhibitor to substrate ratio of about 0.4 giving 50% inhibition<sup>15</sup>, the addition of  $\beta$ -glucosidase to *Trichoderma* cellulase markedly increases the rate and extent of saccharification with a maximum effect at around 3 units per ml<sup>14</sup>. Since means of increasing  $\beta$ -glucosidase in *Trichoderma* fermentations have not been found, addition of  $\beta$ -glucosidase from other microbial sources such as *Aspergillus phoenicis*<sup>11</sup> or *Botryodiplodia theobromae*<sup>19</sup> are being tested with good results.

#### Mutation

Most successful commercial fermentations to produce biological products are based on the use of mutant strains. The wild *Trichoderma* strain has already been mutated to improve the yield approximately 4 fold (Table 2) but further improvements would appear feasible. At present the mutation programme is slow because satisfactory plating assays to screen isolates for enhanced cellulase production have not been developed and because cellulase production

in shake flasks is limited more by the medium than by the strain.

High yields on enriched media require instrumented pH control to give the desired programme of a growth phase at higher pH, followed by an enzyme production phase at around pH 3.5, while preventing a fall to inactivating pH levels. It is possible to buffer media to prevent pH fall below 3.5, but to date such media have not given satisfactory yields of cellulase.

The chief objective of a mutation programme is enhanced cellulase productivity. Other desirable mutants would include constitutive or derepressed mutants which might solve the problems of using enriched media, increased production of extracellular  $\beta$ -glucosidase and production of enzymes more resistant to temperature inactivation and/or sugar inhibition.

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